

インスリン様成長因子Iはマウス子宮内膜間質細胞のDNA合成を促進する

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—Original—

Insulin-Like Growth Factor-I Stimulated DNA Replication in Mouse Endometrial Stromal Cells

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Abstract. Much evidence has suggested that sex steroid hormone-induced growth of uterine cells is mediated by polypeptide growth factors synthesized in uterine tissues. The present study aimed to clarify the effect of insulin-like growth factor-I (IGF-I) on the proliferation of mouse endometrial stromal cells obtained from immature mice. IGF-I and IGF-I receptor (type I) mRNAs were detected in the endometrial stromal cells. IGF-I increased bromodeoxyuridine (BrdU) uptake in the endometrial stromal cells, indicating an increase in DNA replication. E2 increased IGF-I mRNA levels in the endometrial stromal cells. IGF-I receptor is a tyrosine kinase receptor, and treatment with genistein, a tyrosine kinase inhibitor, reduced IGF-I-induced BrdU-uptake in the endometrial stromal cells. IGF-I signaling pathways involve mitogen-activated protein (MAP) kinase and phosphatidylinositol-3 kinase (PI-3 kinase). Treatment with 10^{-7} M of the MAP kinase inhibitor PD098059 and 10^{-5} M of the PI-3 kinase inhibitor LY294002 decreased IGF-I-induced BrdU-uptake in the endometrial stromal cells. However, LY294002 (10^{-5} M) also decreased the BrdU-uptake in the absence of IGF-I treatment. These results suggest that endometrial IGF-I is involved in the proliferation of endometrial stromal cells in a paracrine or autocrine manner, and that the MAP kinase pathway is involved in DNA replication of endometrial stromal cells.

Key words: Endometrium, IGF-I, MAP kinase, PI-3 kinase, Mouse

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The uterine endometrium consists of epithelial and stromal cells, and the growth and function of endometrial cells are regulated by estrogens and progestins [1]. The steroid hormone-induced growth of uterine endometrial cells is mediated by polypeptide growth factors synthesized in uterine tissue [2, 3]. Several reports have indicated that estrogen-induced proliferation of endometrial epithelial cells is mediated by growth factors such as insulin-like growth factor-I (IGF-I) [4, 5], epidermal growth factor (EGF) [6–8] and transforming growth factor- α (TGF- α) [9]. In

endometrial stromal cells, on the other hand, the proliferation is induced by a combined treatment of estradiol and progesterone [1]. Recently, we found that proliferation of endometrial stromal cells induced by estrogen and progestin, is mediated by EGF or TGF- α [3]. However the molecular mechanism of the steroid hormone-induced proliferation of endometrial stromal cells is still unclear.

IGF-I is known as a regulator of proliferation, differentiation and metabolism in various cells. In the uterus, IGF-I is expressed in endometrial luminal and glandular epithelium, stroma and myometrium in rats and mice [4, 10, 11], and uterine IGF-I production is stimulated by estrogen treatment in both rat and mouse uteri [10, 11]. IGF-

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I treatment induces G1 phase progression, and IGF-I is considered to be a progression factor [12]. In mouse uterine endometrial luminal epithelial cells, IGF-I stimulated DNA replication [5]. However, although IGF-I stimulates DNA replication in endometrial cells [13], it is not clear whether IGF-I stimulates mouse endometrial stromal cells.

IGF-I binding to IGF-I receptor (type I) results in autophosphorylation of tyrosine residues on the receptors leading to activation of intrinsic tyrosine kinase. Following autophosphorylation, the activated IGF-I receptors transmit signals through intracellular signal transduction pathways including the mitogen-activated protein (MAP) kinase and phosphatidylinositol-3 (PI-3) kinase pathways [14]. In mouse endometrial cells it is not clear what types of IGF-I signaling pathways participate in the stimulatory regulation of endometrial cell proliferation. The present study was aimed at studying the effect of IGF-I on the DNA replication of endometrial stromal cells and signaling pathways of IGF-I. Genistein, PD098059 and LY294002 were used as inhibitors for tyrosine kinase, MAP kinase and PI-3 kinase, respectively [15–17]. DNA replication was detected by measuring the uptake of 5-bromo-2'-deoxyuridine (BrdU) into the nucleus of endometrial stromal cells.

Materials and Methods

Animals

Female mice, 21 to 23 days old, of the ICR strain (CLEA Japan Inc., Osaka, Japan) were kept in a temperature-controlled animal room with free access to a commercial diet and tap water. All animal experiments were carried out according to the Guidelines for Animal Experimentation of Okayama University, Japan.

Culture

Endometrial stromal cells were dissociated with trypsin (0.1% (w/v); Sigma-Aldrich, St Louis MO, USA) as described in our previous report [3]. Cell viability was assessed by the trypan blue exclusion test (usually more than 95%). The isolated endometrial stromal cells were suspended in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture (DME/F12; Sigma-Aldrich) without phenol red containing dextran-

coated charcoal-treated fetal bovine serum (DC-FBS, 2% (w/v); GIBCO BRL, Life Technologies Inc., Rockville, MD, USA). The cells were seeded at a density of 2.6×10^4 cells/cm² in 6-well multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) for RNA measurement, and in 24-well plates for the detection of DNA replication. These cells were cultured in DME/F12 medium containing 2% DC-FBS for one day, and in serum-free DME/F12 medium containing 1 g/l bovine serum albumin (Fraction V), 10 ng/l glucagon, 0.1 mg/l hydrocortisone, 400 ng/l 3,3'-triiodothyronine, 200 ng/l parathormone, 5 g/l sodium selenite, 100 ng/l insulin, and 10 mg/l transferrin for 2 days. All supplements were obtained from Sigma-Aldrich. The culture was continued at 37 C in a humidified atmosphere of 5% CO₂ and 95% air.

Hormone treatment

Estradiol-17 β (Sigma-Aldrich) was initially dissolved in sterile ethanol and was stocked at concentration of 10 mM (0.03% ethanol). The working solution of E2 was made by diluting the stock solution with culture media. Genistein, LY294002 and PD098059 were obtained from Sigma-Aldrich. Recombinant human IGF-I was from Amersham Pharmacia Biotech (Uppsala, Sweden).

RNA isolation and reverse transcription of messenger RNA

Total RNA was isolated from cultured endometrial stromal cells using the method of Chomczynski and Sacchi [18], and was precipitated with denaturing solution, iso-propanol and 70% ethanol. After treatment with DNase I (Invitrogen Life Technology, CA, USA) at 25 C for 15 min, used to remove genomic DNA, reverse transcription (RT) was performed using Super Script First-Strand synthesis System for RT-PCR (Invitrogen Life Technology). These samples were reverse transcribed at 42 C for 50 min with oligo (dT)_{12–18} primer (0.5 mg/ml), 200 units/ μ l of SuperScript II, 2 μ l of 10 $\times$ buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 2 μ l of 25 mM MgCl₂, 2 μ l of 100 mM DTT, and 1 μ l of 10 mM dNTP. Non-RT samples were treated with DEPC-treated water without SuperScript II reverse transcriptase. To terminate the reaction, the samples were incubated at 70 C for 15 min. To remove the RNA template, these samples were treated with 2 units/ μ l of RNase H at

37 C for 20 min.

RT-polymerase chain reaction analysis (RT-PCR)

One microliter of RT products was used for PCR using TaKaRa Taq (TAKARA BIO Inc., Otsu, Japan). The sequence of each primer was as follows: IGF-I sense, 5'-GCTGGTGGATGCTCTTCAGTT-3', IGF-I antisense, 5'-CTTCTCCTTTCAGCTTCGTTT-3'; IGF-I receptor sense, 5'-TTCTTCTATGTCCCCGCCAA-3', IGF-I receptor antisense, 5'-AGCCTCGTTTACCGTCTTGAT-3'; GAPDH sense, 5'-CTGGAAAGCTGTGGCGTGATG-3', GAPDH antisense, 5'-TGGAAGAGTGGGAGTTGCTGTT-3'. The sizes of the PCR products generated with these primers were 281 bp, 356 bp and 308 bp for IGF-I, IGF-I receptor and GAPDH, respectively.

Conditions for PCR were as follows: incubation at 96 C for 1 min, and each cycle of reactions included denaturation at 96 C for 30 sec, annealing at 58 C for 30 sec and extension at 70 C for 30 sec followed by incubation at 70 C for 7 min for additional extension. IGF-I cDNA fragments were amplified at 26 cycles, IGF-I receptor cDNA fragments were amplified at 27 cycles and GAPDH cDNA fragments were amplified at 20 cycles. The PCR products for IGF-I, IGF-I receptor and GAPDH mRNAs were increased in accordance with the amounts of RT products (two-fold serial dilutions of template cDNAs).

Twelve microliters of PCR products were electrophoresed on 2.0% (w/v) agarose gel, stained with 0.25 µg/ml ethidium bromide, photographed under ultraviolet illumination, and compared with a known standard (0.5 µg of 100-bp DNA Ladder, New England BioLabs Inc. UK) for size determination. The band intensity was quantified by Kodak 1D image software (Version 3.6). The amount of IGF-I mRNA levels was normalized by the amount of GAPDH mRNA levels for each sample. The authenticity of each amplicon was confirmed by sequencing analysis.

Determination of DNA-replicating cells

DNA-replicating cells were detected using a Cell Proliferation ELISA kit (Roche Applied Science, Mannheim, Germany). Endometrial stromal cells were cultured in the culture medium containing 10 mM 5-bromo-2'-deoxyuridine (BrdU). The measurement of BrdU-uptake into nuclear DNA was performed by enzyme-labeled immunoassay

according to the manufacturer's instruction.

Statistical analysis

All experiments were repeated 3–4 times. Data are presented as means + standard errors of means (SEM). Statistical difference was analyzed by one way of analysis of variance followed by Tukey's test.

Results

Detection of IGF-I and IGF-I receptor mRNAs by RT-PCR analysis in cultured mouse endometrial stromal cells

To detect IGF-I and IGF-I receptor mRNA expression in cultured endometrial stromal cells under serum-free conditions, RT-PCR was performed using primer pairs to amplify IGF-I cDNA and IGF-I receptor cDNA (Fig. 1). A single band was obtained in ethidium bromide-stained gels from the amplifications and sequence analysis revealed that these amplified fragments were cDNAs coding each part of mouse IGF-I and IGF-I receptor (data not shown). DNase-treated RNA samples were amplified without RT reaction, and no bands were detected.

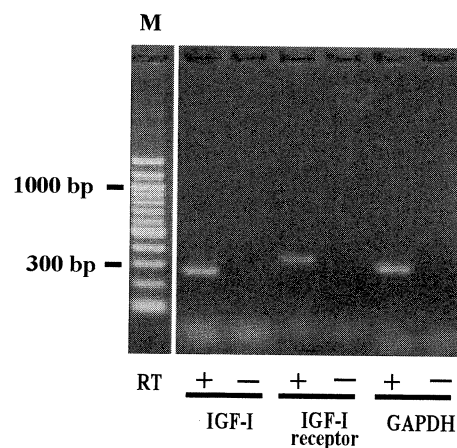


Fig. 1. RT-PCR analysis of IGF-I and IGF-I receptor mRNAs in cultured endometrial stromal cells. Total RNA obtained from the cultured endometrial stromal cells was reverse-transcribed and amplified by PCR using primers for IGF-I, IGF-I receptor and GAPDH cDNAs. As negative controls, RNA samples without RT treatment (RT-) were amplified. Each sample was electrophoresed, and stained with ethidium bromide. M: 100-bp ladder.

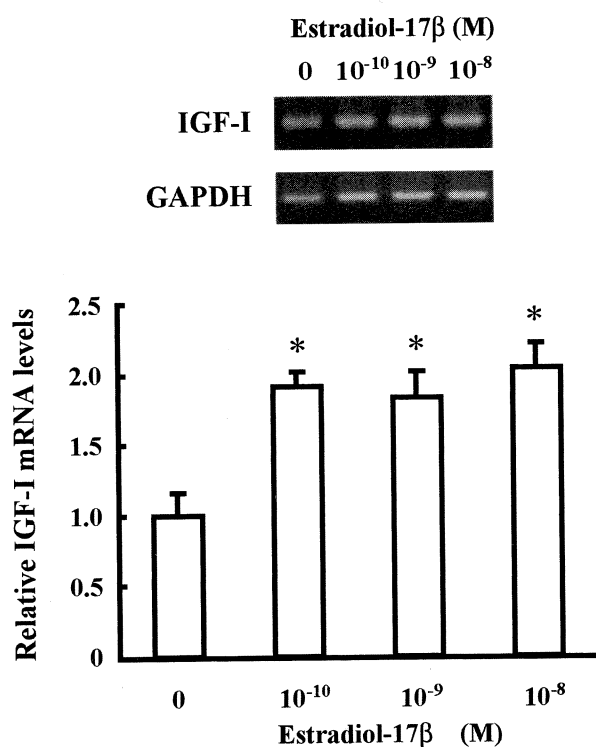


Fig. 2. Effect of E2 on IGF-I mRNA levels in cultured endometrial stromal cells. The cells were treated with E2 (10^{-10} , 10^{-9} and 10^{-8} M) for 24 h. IGF-I mRNA levels were measured by semi-quantitative RT-PCR. Upper photographs show typical ethidium bromide-stained electrophoretic patterns of PCR products of IGF-I and GAPDH cDNAs. The IGF-I mRNA levels were measured by band intensities of PCR products, and normalized according to the GAPDH mRNA levels. Mean and standard error of the mean were calculated from the data of three different preparations of cultured wells. * $p < 0.01$, significantly different from the control (0 M).

Effect of E2 on IGF-I mRNA expression in mouse endometrial stromal cells

E2 treatment (10^{-10} , 10^{-9} and 10^{-8} M) for 24 h significantly increased IGF-I mRNA levels in endometrial stromal cells compared with the control ($P < 0.05$, Fig. 2). Endometrial stromal cells were treated with E2 (10^{-9} M), and IGF-I mRNA was elevated at 6, 18, 24 and 48 h compared with the control (Fig. 3).

Effect of IGF-I on DNA replication of mouse endometrial stromal cells

BrdU-uptake in cultured endometrial stromal cells was measured to study the effect of IGF-I on the DNA replication of endometrial stromal cells.

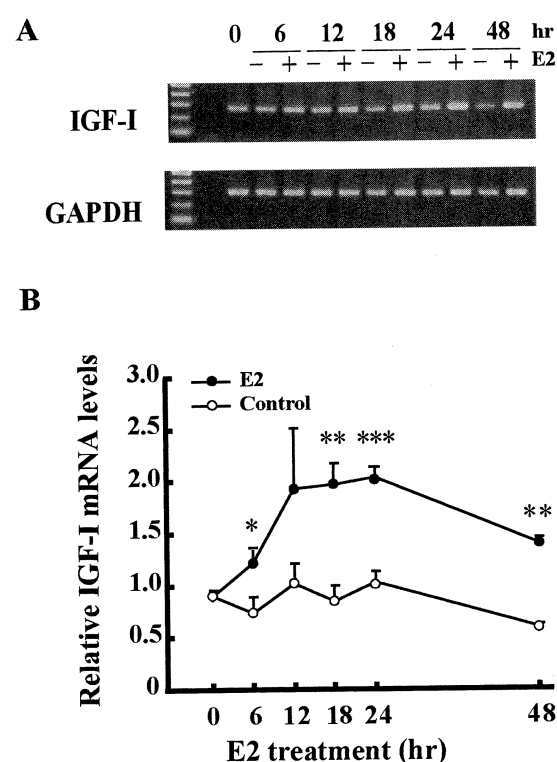


Fig. 3. Time course effect of E2 on IGF-I mRNA levels. Cultured endometrial stromal cells were treated with E2 (+, 10^{-9} M). Controls were without E2 (–). IGF-I mRNA levels were measured by semi-quantitative RT-PCR analysis. **A.** A typical example of ethidium bromide-stained RT-PCR products, the upper photograph represents amplified IGF-I cDNAs, and the lower photograph represents amplified GAPDH cDNAs. **B.** A summary of semi-quantitative RT-PCR analysis of IGF-I mRNA levels. The values represent relative levels of IGF-I mRNA compared with the control, after 24 h without E2 treatment, which was defined as 1.0. Mean and standard error of the mean (SEM) were calculated from the data of three different preparations of cultured wells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; compared with the control (24 h).

IGF-I treatment increased BrdU-uptake into the stromal cells in a dose-dependent manner (Fig. 4) and IGF-I treatment (10^{-8} M) increased BrdU-uptake at 16 and 48 h (Fig. 5).

Effect of genistein, PD 098059 or LY 294002 on IGF-I-induced DNA replication of endometrial stromal cells

To clarify the signaling pathways involved in

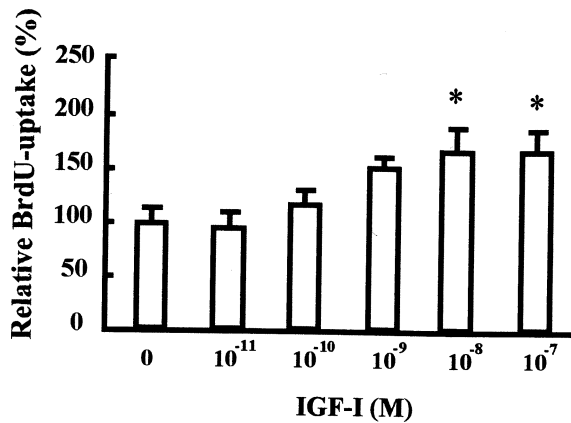


Fig. 4. Effect of IGF-I on the DNA replication of endometrial stromal cells. Cultured endometrial stromal cells were treated with IGF-I (0, 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} and 10^{-7} M), and BrdU (10^{-5} M) for 16 h. The values represent the relative levels of BrdU-uptake compared with the control level (without IGF-I treatment) that was defined as 100%. Each value is the mean + SEM of three or four different cultured wells. * $P < 0.01$, compared with the control.

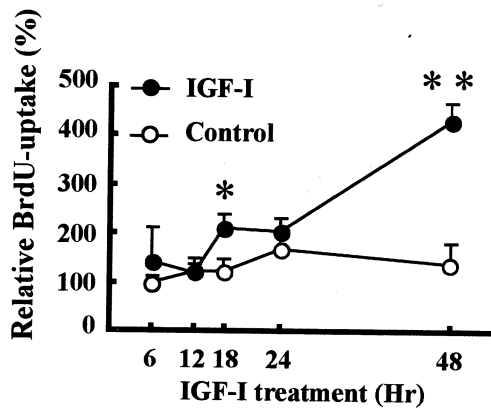


Fig. 5. Time course of IGF-I effect on the DNA replication of endometrial stromal cells. Cultured endometrial stromal cells were treated with IGF-I (0 or 10^{-8} M). BrdU labeling continued throughout the IGF-I treatment. The values represent the relative levels of BrdU-uptake compared with the control levels (without IGF-I treatment) at 6 h that was defined as 100%. The relative levels of BrdU-uptake in IGF-I-treated cells were significantly higher at 16 and 48 h than those in non-treated control cells. Each value is the mean + SEM of three or four different cultured wells. * $P < 0.05$, ** $P < 0.01$ compared with the corresponding control.

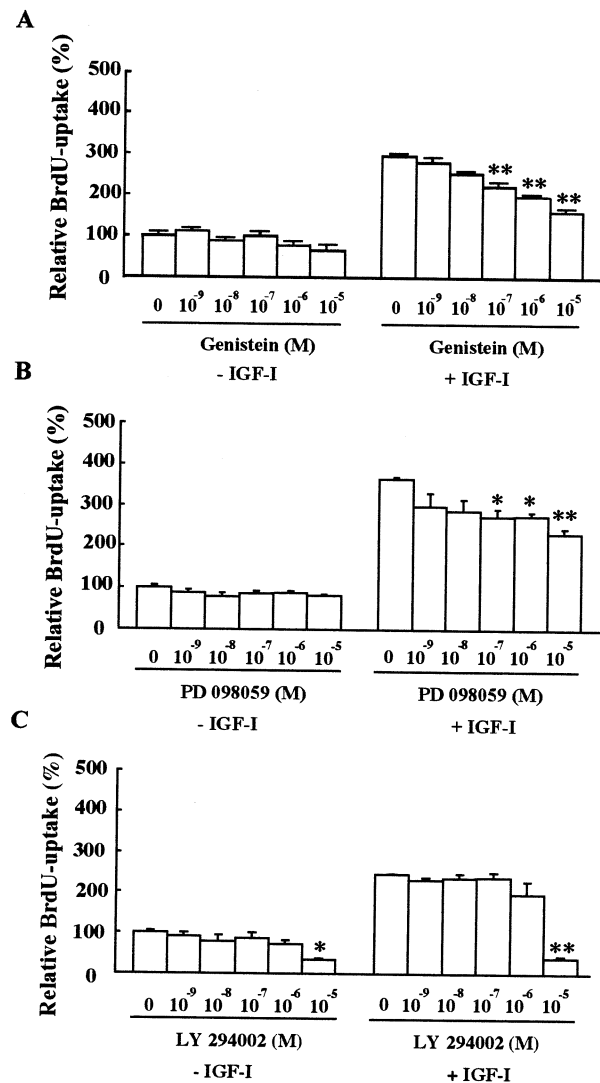


Fig. 6. Effect of genistein, PD098059 or LY294002 on IGF-I-induced DNA replication of endometrial stromal cells. Cultured endometrial stromal cells were treated with combination of IGF-I (0, or 10^{-8} M) and genistein PD098059 or LY294002 for 16 h. BrdU-uptake was measured by an enzyme-labeled immunoassay. The values represent relative levels of BrdU-uptake compared with the control without the treatment that was defined as 100%. Each value is the mean + SEM of three or four different cultured wells. * $P < 0.05$, ** $P < 0.01$ compared with the IGF-I-treated control (0 M of each inhibitor).

IGF-I-induced DNA replication of endometrial stromal cells, cultured endometrial stromal cells were treated with genistein, PD098059 or LY294002 in the presence of IGF-I (Fig. 6). Genistein and PD098059 reduced IGF-I (10^{-8} M) induced BrdU-

uptake into the endometrial stromal cells in a dose-dependent manner. Genistein and PD098059 both showed inhibitory action at the minimum dose of 10^{-7} M. LY294002 (10^{-5} M) reduced the stimulatory effect of IGF-I on BrdU-uptake, and in the absence of IGF-I treatment significantly decreased BrdU-uptake, although genistein and PD098059 did not affect BrdU-uptake.

Discussion

The results of several studies suggest that IGF-I produced in endometrial cells is essential for estrogen-induced proliferation of endometrial epithelial cells [5, 11, 13, 19]. On the other hand, Sato *et al.* [20] recently reported that systemic IGF-I is involved in growth of the uterus, and local IGF-I is not a direct mediator of estrogen action in the endometrial epithelial cells. The present study demonstrated that DNA replication of cultured endometrial stromal cells obtained from immature mice is stimulated by IGF-I treatment. IGF-I mRNA was detected in the cultured endometrial stromal cells, which is in agreement with previous studies which analyzed IGF-I mRNA in human, rat and mouse uteri [10, 21–23]. IGF-I receptor mRNA was detected in the cultured endometrial stromal cells, and previous studies have reported that IGF-I receptor mRNA is mainly localized in luminal and glandular epithelial cells in adult uteri [21, 22, 24], and also in stromal cells, although the amounts were less than in endometrial epithelial cells [21]. Expression of IGF-I receptor mRNA might change during postnatal development, particularly at the peripubertal period. However, these findings together indicate that uterine IGF-I regulates functions of luminal and glandular epithelial cells, and stromal cells in an autocrine or paracrine manner.

Estrogen increased IGF-I mRNA levels in cultured endometrial cells. Previous studies have reported the stimulatory effect of estrogen on IGF-I gene expression [4, 11, 25]. Most studies have used the whole uterus consisting of endometrium, myometrium and other tissues to study the IGF-I mRNA expression, however, in the present study we examined IGF-I gene expression in endometrial stromal cells isolated from the uterus. The stimulatory effect of estrogen on IGF-I mRNA levels was evident at 6 h after E2 treatment. Our *in*

vitro data concerning the estrogen effect correspond well to *in vivo* data obtained from ovariectomized rats [4]. It is unclear whether E2 stimulated IGF-I gene transcription or prolonged the stability of IGF-I mRNA, however, it is probable that increases in IGF-I mRNA levels result in enhanced IGF-I synthesis, leading to stimulation of DNA replication of cultured endometrial stromal cells.

Ghahary *et al.* [10] demonstrated the DNA synthesizing activity of IGF-I on uterine cells in an organ culture system. Beck *et al.* [13] also showed the stimulation of IGF-I on DNA replication of cultured uterine cells, although they did not identify the cell types. In the present study we showed that IGF-I treatment stimulated the proliferation of cultured endometrial stromal cells. As discussed above, endometrial stromal cells expressed IGF-I receptor mRNA in our culture system, and IGF-I is thought to act on stromal cells through IGF-I receptors, thus stimulating DNA replication.

IGF-I affects cell proliferation and apoptosis [14]. In cultured endometrial epithelial cells, IGF-I stimulates cell proliferation [5]. The results of the present study indicate that IGF-I stimulates DNA replication of cultured endometrial stromal cells in serum-free conditions under continuous exposure to IGF-I and BrdU. The first increase in BrdU-uptake was observed at 16 h (1.7-fold above the control level). At 24 h the BrdU-uptake in the IGF-I-treated group did not differ from that in the control group. A second increase in BrdU-uptake was observed at 48 h (1.7-fold above the control level). The first increase in BrdU-uptake might correspond to an initial increase in the number of DNA-replicating cells in response to IGF-I treatment, and the following increase at 48 h might correspond to a second wave of DNA-replication. These findings suggest that IGF-I stimulates DNA replication, accelerating the cell cycle of endometrial stromal cells.

It is well known that IGF-I is a progression factor in the cell cycle, and it is associated with G1 phase progression and DNA replication [12]. IGF-I is considered a mediator of estrogen-induced growth of endometrial epithelial cells [4]. Recently, Adesanya *et al.* [19] reported that IGF-I is a G2 progression factor in estrogen-induced proliferation of endometrial epithelial cells. We found, however, that in endometrial stromal cells IGF-I stimulated DNA replication in a dose-

dependent manner, and significant stimulation was detected at 16 h after IGF-I addition. Consequently, the possibility that IGF-I is a G1 progression factor in endometrial stromal cells can not be entirely excluded. It is probable that the cell-cycle regulatory mechanism might differ between epithelial and stromal cells, since the DNA replication of endometrial epithelial cells requires the presence of endometrial stromal cells [2], but that of endometrial stromal cells might not require the presence of endometrial epithelial cells.

IGF-I receptors have a tyrosine kinase activity at the intracellular domain, and IGF-I binding leads to autophosphorylation of these receptors. Activated IGF-I receptors phosphorylate tyrosine residues of substrate molecules. IGF-I signaling is mediated through several pathways, the two major pathways being the MAP kinase and PI-3 kinase pathways [14]. In human neuroblastoma cell line SH-SY5Y cells, MAP kinase and PI-3 kinase pathways are required for IGF-I-induced cell proliferation [26]. Similarly, IGF-I-induced DNA replication of intestinal smooth muscle cells occurs through MAP kinase and PI-3 kinase pathways [27]. In addition, rat fibroblasts [28], rat fetal brown adipocytes [29], and 3T3-L1 preadipocytes [30] were stimulated by IGF-I through the MAP kinase pathway. In breast cancer MCF-7 cells, IGF-I down-regulates progesterone receptor gene expression through the PI-3 kinase pathway [31]. In mouse endometrial stromal cells, genistein and PD098059 blocked IGF-I-induced DNA replication, suggesting that the MAP kinase pathway is involved in IGF-I-induced DNA replication. PD098059 treatment was not as effective as genistein. Genistein probably blocks tyrosine kinase activity of IGF-I receptors, which are located in up-stream of the MAP kinase pathway. On the other hand, LY294002 decreased BrdU-uptake in IGF-I-treated stromal cells and even in non-treated stromal cells. This inhibitory effect of LY294002 required a higher dose (10^{-5} M) than that of PD098059 (10^{-7} M). This suggests that the inhibitory effect of LY294002 might be ascribable to its pharmacological effect on stromal

cells. The possibility still remains that the PI-3 kinase pathway is involved in IGF-I-induced DNA replication. Further analysis is needed to clarify the involvement of PI-3 kinase pathway in IGF-I-induced DNA replication.

The IGF binding protein (IGFBP) gene family consists of six members encoding a family of multifunctional proteins, IGFBP-1 to IGFBP-6. A major function is IGF transport, mainly exerted by IGFBP-3. In addition to the IGFBP transport functions, IGFBPs are involved in cell cycle, apoptosis, cell adhesion and motility in various tissues [32, 33]. IGFBP expression in the uterus is highly associated with IGF-I activity [34–36]. In the human endometrium, the expression of mRNAs for the six IGFBPs are detected during the menstrual cycle. IGFBP-1 mRNA is not detected in the proliferating endometrium but it is found in the stromal cells during the secretory phase. IGFBP-1 and IGFBP-3 mRNAs are located in subpopulations of endometrial cells, and IGFBP-2, -4, -5 and -6 mRNAs are diffusely distributed in the stroma [21]. In the rat and mouse uteri six IGFBPs have been detected, and expression of IGFBP mRNAs varies during the perinatal period and the estrous cycle [23, 24, 37]. Uterine IGFBP production is modulated by estrogen [38, 39]. Further analysis of the estrogen effect on the IGF system including IGFBPs in cultured endometrial stromal cells is needed.

In conclusion, mouse endometrial stromal cells expressed IGF-I mRNA, and this expression was regulated by estrogen. IGF-I stimulated DNA replication of endometrial stromal cells, but the DNA replication was inhibited by genistein, and PD098059, suggesting that IGF-I signaling was mediated through the MAP kinase pathway.

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